Experimental Studies in Whole Animal Bioassay*

Animal models have been used for a number of purposes in metal carcinogenesis studies: (a) to detect carcinogenic activity; (b) to estimate carcinogenic risk; and (c) to investigate mechanisms of metal carcinogenesis.

The design of animal tests will be determined by the purpose of the study. In this section we shall distinguish between tests in animals designed to identify carcinogenic activity versus tests intended to have special relevance to the evaluation of effects in man.

Tests of Carcinogenic Activity

General Principles

Guidelines for decisions relative to the carcinogenicity of a metal in experimental animals will include the determination of the purity, the speciation, and the physical state of the metal compound being evaluated in the experimental animals (1).

At the present state of knowledge, short-term or *in vitro* tests are valuable research tools for metal carcinogenesis studies, e.g., molecular mechanisms, but no test or battery of tests has been sufficiently validated to be used reliably in predicting the carcinogenic activity of metal compounds in animals. Tests in whole animals are necessary for the experimental identification of carcinogenic activity.

The intramuscular route of administration has been used to study the carcinogenic potential of

numerous metal compounds. Tumors appear at the site between 4 to 11 months (a few first appear after 12 months). The subcutaneous route is the second most frequent one by which positive results were obtained with metals. Solid-state carcinogenesis may complicate the interpretation of tumors which appear at the site of implantation by this route. Occurrence of tumors only at the site of injection should be followed by studies employing other routes of administration.

Intratracheal instillation has been employed to study the carcinogenic or enhancing effects of several metal compounds. Larger doses can be administered, and maximum tolerated dose can be approximated by this route of administration.

The oral route has been traditionally used for carcinogenic studies of nonmetals. This route has limitations for metal studies since some metals are poorly absorbed from the gastrointestinal tract. Diet and water must be analyzed for metal content. Most commercial rodent diets are high in calcium, zinc phosphates, and phytates, which interfere with metal absorption. The metal being studied must also be analyzed in the control diet. Blood and urine levels of the agent under test must be measured to obtain some indication of the amount of the metal absorbed.

Special routes of administration may be used to answer specific questions, such as the intrathoracic route to induce mesotheliomas.

It must be recognized that, although a metal may be carcinogenic in one or more species or strains, e.g., in rats, mice, hamsters, no generalization can be made about interspecies comparison. Species differences in relation to metal metabolism can have profound effects on the outcome of experiments. From a practical viewpoint, rodents are the animals of choice, but more research is needed to determine the carcinogenic response of other orders. Bioassays on more than one species are

necessary to reduce the possibility of missing the carcinogenic action of a metal.

A major area of research which has been neglected is transplacental and perinatal carcinogenesis by metals. Here the experiments must be so designed as to differentiate intrauterine exposure, neonatal exposure, and exposure through the maternal milk.

Relation to *in Vitro* Studies

Presently, short-term *in vitro* bioassays are under development for detection of metals as carcinogenic agents via both genetic and nongenetic pathways. Current research efforts directed toward improvement of *in vitro* techniques for the detection of metal carcinogens continue to rely heavily on information obtained from *in vivo* bioassay. It is expected that, with further development of *in vitro* bioassays, complex mixtures and key elemental interactions may be screened *in vitro* prior to *in vivo* testing. Furthermore, because of the flexibility of *in vitro* bioassays, such systems may provide clues on how to conduct *in vivo* tests in the most efficient manner. A major difference, however, between *in vitro* systems and the *in vivo* assays relates to chemical speciation. *In vivo* response is markedly dependent on chemical form, i.e., Ni_3S_2 vs. NiS vs. $NiSO_4$, and low and high temperature forms of BeO. Understanding of the effects of different chemical species of metals, however, will provide insight into the mechanism of metal carcinogenesis.

Estimation of Carcinogenic Risk

Comparisons of Carcinogenic Activity

Carcinogenic activity in animal experimentation relates to the dose that causes a given frequency and incidence of cancer under controlled experimental circumstances, species, strain, age, and sex of the experimental animals. The ability to detect carcinogenic activity is also determined by number of animals, positive and negative controls, period of observation, and so forth. Carcinogenic activity is a relative term and implies a comparison with other chemicals such as weak versus strong carcinogens (2).

Measurements of carcinogenic activity are used for experimental purposes, for example, in studies of structure-activity relationships, identifying the "proximate" carcinogen, or assessing the effects of modifying factors on the carcinogenic response. Measurement of weak versus strong carcinogenic

substances in experimental animals have been extrapolated as estimated of relative risks in the human populations for exposures to one substance versus another.

Relevance of Animal Tests to Man

In order to predict hazard to man from animal studies, there must be judicious selection of test species, routes of exposure, and dose. Tests such as *in vitro* tests and/or *in vivo* tests such as intramuscular or subcutaneous injection may or may not precede the use of more relevant exposure techniques.

Selection of Test Animals. Because of ease of handling, accumulated long-term experience, and the capability of the investigator to manipulate fairly large groups of animals, it is likely that the animals chosen for whole animal tests will, in most instances, be standard laboratory rodents, that is, mice, rats, and hamsters. It is essential, however, to recognize the possible shortcomings of this approach. Absorption, transport, storage, excretion, and qualitative and quantitative aspects of biotransformation of metal compounds may vary considerably among rodents and between rodents and humans. Similarly, differences in interaction with target macromolecules and differences in repair capability may be significant determinations of variations in response.

The use of two or more test species will reduce the likelihood of confusing species-specific responses from those applicable to man. Certainly, differences in response among rodent species should be exploited to illuminate the mechanisms of carcinogenesis.

Routes of Administration. An experimental protocol designed to assist in the estimation of human risk should preferentially employ routes of administration identical to or as similar as possible to those involved in human exposure. The route chosen will determine the effect of the test material on tissues immediately contacted as well as on tissues ultimately reached by absorbed and metabolically altered products. Thus, feeding experiments alone are not sufficient to examine systemic carcinogenicity of a material poorly absorbed through the intestine as is the case with many metal compounds. Some metals, although poorly absorbed, may nevertheless accumulate over a lifetime.

Dose. It has become customary to utilize the estimated "maximum tolerated dose" in the design of initial bioassays in animals. The justification for this choice has been the necessity to explore the full

range of effects of a test agent and also the limitation of detectable effects imposed by use of a relatively small group of animals. These should be considered preliminary studies to demonstrate the carcinogenic potential of the substance. However, extrapolation of findings to long-term, lower level exposure in larger groups of humans may require studies at different dose levels. For example, high dose metabolism may differ significantly from metabolism of lesser quantities of metal compounds. High doses, not inconsistent with those occasionally encountered by humans, may produce severe interfering toxic effects. Accordingly, the experimental design should include lower doses that do not produce such toxicity. Patterns of dosage may include such things as intermittent exposure, short-term high level exposure followed by prolonged observation, or even single dose exposure, particularly to refractory materials with long biological half lives.

Careful attention must be given to those determinants of dose other than total quantity. These include such factors as particle size, solubility, stability, and the metabolic pathways which determine ultimate dose to the target tissue and cell.

Comparison of Animal Studies with Epidemiological Findings in Man.

Bioassay of metal compounds in experimental animals may be predictive of effects in man. Although there are a number of quantitative differences between various species of laboratory animals and man, there is a basic universality of biological systems that permits extrapolation of effects in animals to man (3-5). Ideally, animal studies should provide sufficient information regarding the carcinogenic potential of metal compounds to support regulatory measures that are protective to human health. In those instances where human effects have already occurred or are suspected, animal studies may confirm the activity of a specific substance and route of exposure and may also elucidate mechanisms and factors that influence effects in man.

To date, exposures to arsenic, beryllium, cadmium, chromium, and nickel (or their compounds) have been identified as contributing to the development of human cancer (6). Certain compounds of beryllium, nickel, and chromium, which are accepted as contributing to pulmonary cancer in man, also produce tumors in animals exposed via the respiratory tract. Animal models treated by similar routes of exposure produce tumors with compounds of these metals.

Arsenic has been linked through epidemiologic studies to skin cancers following ingestion of As in drinking water and Fowler's solution and to carcinoma of the lung in workers exposed by inhalation (7). In spite of extensive investigation in diverse animal species, there is no definite evidence that arsenic is carcinogenic for experimental animals. Recent studies, however, involving intratracheal administration of a mixture containing arsenic, copper sulfate, and calcium oxide resulted in pulmonary tumors (8). These findings point to differences of susceptibility to arsenic compounds between man and certain experimental animals. Also, the epidemiologic studies may reflect other mixed exposures of which arsenic is only one component of exposure to specific forms of inorganic arsenic. Prostatic cancer has not been found following administration of cadmium by gastric gavage or injection, but these studies resulted in low tissue content of metal (9). There have been no reported long-term studies of inhalation of cadmium compounds in animal models for carcinogenesis. Intramuscular and subcutaneous injection resulted in sarcomas at the site of injection. Testicular tumors were found after systemic intratesticular injection in fowl.

A number of metal compounds have been reported to cause tumors in experimental animals, but corresponding carcinogenicity has not been established by studies in man. Cobalt, manganese, titanium, zinc, and carbohydrate-iron compounds each produce tumors by parenteral routes at the site of injection, a route of exposure not likely to occur in man (10). Such studies by injection routes may, however, provide incentive for further studies employing other routes of exposures. Also, such studies may be predictive of implant effects, perhaps relative to usage of materials for prosthesis, although the role of physical factors as well as the chemical nature of the metal compound must be considered in such evaluations.

Lead is the only metal to date that produced tumors in rats following oral administration (11). There are some epidemiological data regarding the carcinogenicity of lead in man (see Epidemiology Report). These data, however, do not provide sufficient evidence to support definite conclusions as to whether lead causes human cancer (12).

Differences in susceptibility to toxicity between man and experimental animals may permit levels of exposure to the metal in experimental animals that cannot be tolerated in man, thus leading to discrepancies between epidemiological observations and animal studies.

It is concluded that the relevance of animal studies to man is closer when the same metal compound produces tumors by similar routes of exposure in man and animal models, produces tumors in common target organs, and produces tumors in different animal orders.

Mechanisms of Metal Carcinogenesis

Route of exposure appears to be an important factor in metal carcinogenesis. It is generally difficult to produce tumors by adding metals to food or water, since absorption from the gastrointestinal tract is often poor.

Metabolism is an important aspect in considering mechanisms of metal carcinogenesis. Quantification of initial deposition of the metal in target tissues (lung, skin), eventual translocation to susceptible tissues (bone, kidney), and persistence of metals within their target are important elements in understanding and possibly predicting mechanisms.

A substantial amount of data indicates that solubility, valence state, oxidation-reduction reactions, and formation of complexes are important determinants in metal carcinogenesis (13). It will not be adequate to obtain in vivo data on the behavior of metallic cations only. Experimental approaches need to be developed which allow the study of possible biotransformation and behavior of metals in complex biological surroundings.

It is the prevailing hypothesis that some metals cause cancer by inducing damage directly to the genome (14-18). The evidence has been summarized in recent reviews (1, 19). Less data from whole animal studies are available to support the hypothesis. Such information should become available. However, it would appear important that the experimental model be carefully selected. Unless a given experimental approach will produce a high tumor response in vivo, it will not be possible to link, with confidence, molecular or cellular changes directly to the eventual development of cancer.

Metal compounds may also produce tumors through nongenetic mechanisms. One notion is that metals are promoters. Furthermore, their effects on intracellular metabolism make cells more susceptible to initiation. Certain platinum compounds have been shown to be carcinogenic in animals and to act as initiators for the mouse skin (20). There is a large amount of information available on how metals inhibit enzymes, bind to cellular macromolecules other than DNA (nuclear proteins, RNA), bind to receptors or interfere with membrane functions. Some of these adverse effects are quite specific for either a particular enzyme or for a particular metal. They often occur at metal concentrations which are orders of magnitudes below those found to induce genetic damage. In at least some circumstances, metal carcinogenesis may be mediated by trophic hormone effects. A possible example is induction of Leydig cell tumors in rats following testicular necrosis after subcutaneous injection of $CdCl_2$ (21).

The relation of immune response to metal carcinogenesis has received too little attention thus far. Certain metals (e.g., Pb) appear to suppress the general immunocompetence of the host, but other metals (e.g., Be) were shown to have allergenic properties. With beryllium, inhalation exposure has suppressed a previously induced cutaneous hypersensitivity to the same ionic species of the metal (22).

Metal interactions in carcinogenesis have recently been reviewed (23-25). Interactions may be divided into the following areas: (a) metal antagonism or synergism with metal carcinogens; (b) metal antagonism or synergism with organic chemical carcinogens; and (c) biological factors affecting the expression of chemical carcinogenesis. A major limitation in interpreting the health significance of co-exposure from complex mixtures is the lack of specific information on the chemical form of the biologically active element.

Clearly, from a toxicological and nutritional standpoint, metal-metal interactions are well documented, i.e., Zn-Cd, Ca-Zn, Cu-Zn, Se-Hg, Se-Cd, etc. Few carcinogenesis studies, however, have examined such interactions. Co-exposure of zinc acetate and cadmium chloride (21) or manganese metal and nickel subsulfide (26) have resulted in substantial reductions in tumor incidence. Metabolic studies of the Mn-Ni system have not elucidated the mechanism of antagonism. Thus, further work is required to evaluate the carcinogenicity of co-exposures. Furthermore, in light of the increasing evidence of varied essential and nonessential elemental exposure in human populations, nutritional status must be considered in the evaluation of the totality of human exposures to trace elements.

Trace element exposure often occurs in combination with organic chemical carcinogens. Inhibition of carcinogenesis has been described with copper and selenium compounds. Azo-dye hepatocarcinogenesis may be entirely suppressed by dietary copper supplementation probably due to more rapid catabolism via azo reduction (27). On the other hand, copper protection against ethionine induced hepatomas is thought to be due to decreased catabolism and increased hepatotoxicity (28). Such studies demonstrates the potential complexity of metal interactions.

The best evidence of synergism results from studies of respiratory tract co-exposure to inorganic particles and organic carcinogens (29). Although the mechanism is not completely understood, rate of release of carcinogen and particle size appear to be important factors in the incidence of respiratory tract tumors. Arsenic activity might be related to exposures of mixtures. However, the

role of arsenic as a cocarcinogen is unclear. Recent studies indicate an important role for trace elements as regulators of the immune system as well as activators of viral expression. For example, dietary zinc status may alter the growth rate and incidence of metastases of transplanted tumors (*30*).

Recommendations

1. The investigation of metabolic conversions involving oxidation states *in vivo* and *in vitro* and the relationship to biological effects should be studied.
2. The development and improvement of analytical methods should be undertaken to identify biologically active chemical species.
3. The investigation of the role of immune response to metal compounds and metal hypersensitivity for metal carcinogenesis should be examined.
4. Investigation of the role of metals as cocarcinogens and promoters need to be investigated. Animal as well as *in vitro* models are required for examination of possible promoting activity for metal compounds.
5. Analytical techniques for speciation of metal compounds in complex mixtures (e.g., coal fly ash) and in body tissues should be developed.
6. There should be consideration of more research on the carcinogenic response of animals other than rodents, such as avian and aquatic types (*10, 11*).
7. Further studies of carcinogenesis resulting from perinatal and transplacental exposure are required.
8. The synergism and antagonism between metal compounds, particles and organic chemical carcinogens, and the underlying mechanisms, should be investigated.
9. Methods relevant to the carcinogenesis testing of alloys and metallic substances that are being used for prostheses for dentistry, orthopedics and for artificial internal organs should be developed.
10. The role of chemical and physical forms in their carcinogenicity should be investigated for selected metals.
11. The assessment of carcinogenic activity in an animal model which suggests the possibility that a metal compound may have a cancer inhibitive or an anticarcinogenic effect should be carefully defined and given emphasis in the planning of further investigations. The possibility that selenium and zinc in certain circumstances may have anticarcinogenic effects requires further investigation.

REFERENCES

1. Furst, A. Bioassay of metals for carcinogenesis—whole animals. Environ. Health Perspect. 00: 000 (1981).
2. Searle, C. E. Chemical Carcinogen. ACS Monograph 173, American Chemical Society, Washington, D.C., 1976, pp. xiv-xvi.
3. Tomatis, L. The predictive value of rodent carcinogenicity tests in the evaluation of human risks. Ann. Rev. Pharmacol. Toxicol. 19: 511 (1979).
4. Crouch, E., and Wilson, R. Interspecies comparison of carcinogenicity potency. J. Toxicol. Environ. Health 5: 1095 (1979).
5. Rall, D. P. Validity of extrapolation of results of animal studies to man. Ann. N.Y. Acad. Sci. 329: 85 (1979).
6. Workshop report. Problems of epidemiological evidence. Environ. Health Perspect. 40: 11 (1981).
7. Pershagen, G. Carcinogenicity of arsenic. Environ. Health Perspect. 40: 93 (1981).
8. Ivankovic, S., Eisenbrand, G., and Preussman, R. Lung carcinoma induction in BD rats after a single intratracheal instillation of an arsenic-containing pesticide mixture formerly used in vineyards. Int. J. Cancer 24: 786 (1979).
9. Piscator, M. Role of cadmium in carcinogenesis. Environ. Health Perspect. 40: 107 (1981).
10. Kazantzis, G. Role of cobalt, iron, lead, manganese, mercury, platinum, selenium, and titanium in carcinogenesis. Environ. Health Perspect. 40: 143 (1981).
11. Van Esch, G. J., and Kroes, R. The induction of renal tumors by feeding basic lead acetate to mice and hamsters. Brit. J. Cancer 16: 289 (1962).
12. Kang, H. K., Infante, P. F., and Carra, J. S. Occupational lead exposure and cancer. Science 207: 934 (1980).
13. Jennette, K. W. Role of metals in carcinogenesis: biochemistry and metabolism. Environ. Health Perspect. 00: 000 (1981).
14. Workshop Report. *In vitro* models and methods for bioassay and studies of cellular mechanisms. Environ. Health Perspect. 40: 35 (1981).
15. Vainio, H., and Sorsa, M. Chromosome aberrations and their relevance to metal carcinogenesis. Environ. Health Perspect. 40: 173 (1981).
16. Rossman, T. Effect of metals on mutagenesis and DNA repair. Environ. Health Perspect. 40: 189 (1981).
17. Sirover, M. A. Effects of metals in *in vitro* bioassays. Environ. Health Perspect. 40: 163 (1981).
18. Zakour, R. A., Kunkel, T. A., and Loeb, L. A. Metal induced infidelity of DNA synthesis. Environ. Health Perspect. 40: 197 (1981).
19. Sunderman, F. W., Jr. Mechanisms of metal carcinogenesis. Biol. Trace Element Res. 1: 63 (1979).
20. Leopold, W. R., Miller, E. C., and Miller, J. A. Carcinogenicity of antitumor *cis*-platinum (II) coordination complexes in the mouse and rat. Cancer Res. 39: 913 (1979).
21. Gunn, S. A., Gould, T. C., and Anderson, W. A. D. Cadmium-induced interstitial cell tumors in rats and mice and their prevention by zinc. J. Natl. Cancer Inst. 31: 745 (1963).
22. Reeves, A. J., Krivanek, N. D., and Busby, E. K. Immunity

to pulmonary berylliosis in guinea pigs. Int. Arch. Arbeitsmed. 29: 209 (1972)

23. Sunderman, F. W., Jr. Carcinogenicity and anticarcinogenicity of metal compounds. In: Environmental Carcinogenesis, P. Emmelot and E. Kriek, Eds., Elsevier/North Holland Press, Amsterdam, 1979, pp. 165-192.

24. Fisher, G. L . Trace element interactions in carcinogenesis. In: Trace Metals in Health and Disease, N. Kharasch, Ed., Raven Press, New York, 1979, pp. 93-107.

25. Nordberg, G. F., and Andersen, O. Metal interactions in carcinogenesis: enhancement, inhibition. Environ. Health Perspect. 40: 65 (1981).

26. Sunderman, F. W., Jr., Kasprzak, K. S., Lau, T. J., Minghetti, P. O., Maenza, R. M., Becker, N., Onkelinx, C., and Goldblatt, P. J. Effects of manganese on carcinogenicity and metabolism of nickel subsulfide. Cancer Res. 36: 1790 (1976).

27. Yamane, Y., Sakai, K., Uchiyama, I., Tabata, M., Taga, N., and Hanaki, A. Effect of basic cupric acetate on the biochemical changes in the liver of the rat fed carcinogenic aminoazo dye. I. Changes in the activities of DAB metabolism by liver homogenate. Chem. Pharm. Bull. 17: 488 (1969).

28. Brada, Z., Altman, N. H., and Bulba, S. The effect of cupric acetate on ethionine metabolism. Cancer Res. 35: 3172 (1975).

29. Saffiotti, U . Experimental respiratory tract carcinogenesis and its relation to inhalation exposures. In: Inhalation Carcinogenesis, M. G. Hanna, P. Nettesheim, and J. R. Gilbert, Eds., USAEC, Washington, D.C., 1970, pp. 23-54.

30. Dewys, W., and Pories, W. J. Inhibition of a spectrum of animal tumors by dietary zinc deficiency. J. Natl. Cancer Inst. 48: 375 (1972).